1-1-2010

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Plant Biotechnology Journal

Plant Biotechnology Journal (2010) **8**, pp. 332–350 doi: 10.1111/j.1467-7652.2009.00486.x

Chloroplast-derived enzyme cocktails hydrolyse lignocellulosic biomass and release fermentable sugars

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Received 13 June 2009;
revised 2 August 2009;
accepted 20 October 2009.
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Summary

It is widely recognized that biofuel production from lignocellulosic materials is limited by inadequate technology to efficiently and economically release fermentable sugars from the complex multi-polymeric raw materials. Therefore, endoglucanases, exoglucanase, pectate lyases, cutinase, swollenin, xylanase, acetyl xylan esterase, beta glucosidase and lipase genes from bacteria or fungi were expressed in *Escherichia coli* or tobacco chloroplasts. A PCR-based method was used to clone genes without introns from *Trichoderma reesei* genomic DNA. Homoplasmic transplastomic lines showed normal phenotype and were fertile. Based on observed expression levels, up to 49, 64 and 10, 751 million units of pectate lyases or endoglucanase can be produced annually, per acre of tobacco. Plant production cost of endoglucanase is 3100-fold, and pectate lyase is 1057 or 1480-fold lower than the same recombinant enzymes sold commercially, produced via fermentation. Chloroplast-derived enzymes had higher temperature stability and wider pH optima than enzymes expressed in *E. coli*. Plant crude-extracts showed higher enzyme activity than *E. coli* with increasing protein concentration, demonstrating their direct utility without purification. Addition of *E. coli* extracts to the chloroplast-derived enzymes significantly decreased their activity. Chloroplast-derived crude-extract enzyme cocktails yielded more (up to 3625%) glucose from filter paper, pine wood or citrus peel than commercial cocktails. Furthermore, pectate lyase transplastomic plants showed enhanced resistance to *Erwina* soft rot. This is the first report of using plant-derived enzyme cocktails for production of fermentable sugars from lignocellulosic biomass. Limitations of higher cost and lower production capacity of fermentation systems are addressed by chloroplast-derived enzyme cocktails.

Keywords: biofuel, renewable energy, cellulosic ethanol, cell wall-degrading enzymes, fermentable sugars, lignocellulosic biomass.

Introduction

Plant cell wall is the major component of lignocellulosic biomass which provides abundant renewable polysaccharides in nature. Because of changing global energy needs and finite petroleum reserves, there is an urgent need to develop technologies for harnessing renewable energy resources. The US Congress 'Energy Independence and Security Act of 2007' set the goal for annual production of 16 billion gallons of cellulosic ethanol by 2022. European Union's requirement that 10% of all transport fuels come from renewable sources makes this a global challenge (Robertson *et al.*, 2008). The major biofuel in use today is corn-derived ethanol. In the US, 25%–30% of corn production is currently used for ethanol production. Increasing infrastructure investment in grain ethanol production will consume a substantial portion of corn production (Robertson *et al.*, 2008) raising prices of corn and other food/feed sources. Corn ethanol has been reported to produce more greenhouse gas emission than gasoline. On the other hand, cellulosic ethanol from nonfood crops and from waste produce much less

greenhouse gas emission, even less than electricity or hydrogen, the two energy sources that are thought to be important for solving the problem of greenhouse gas emission (Charles, 2009). Therefore, renewable lignocellulosic biomass from agricultural wastes and wood products is a very attractive feedstock for bioethanol production (US DOE, 2007).

Pectate lyases (EC 4.2.2.2) play an important role in degrading pectic polysaccharides that are important components of primary cell wall of plants (Carpita and Gibeaut, 1993). Pectate lyase randomly cleaves α-(1-4) linkages between galacturonosyl residues, generating 4,5-unsaturated oligogalacturonates (OG) by β-elimination (Yoder *et al.*, 1993). They have been extensively studied in plant pathogens and the action of these enzymes results in the maceration of plant tissues leading to pathogenesis (Collmer and Keen, 1986; Crawford and Kolattukudy, 1987; Herron *et al.*, 2000; Lietzke *et al.*, 1994). Many pathogens like *Erwinia*, *Fusarium*, *Clostridium* and *Bacillus* produce pectate lyases that are involved in the degradation of pectic compounds. Pectic compounds of plant cell wall are primarily made of α-1,4 linked polygalactosyluronic acid residues interspersed with regions of alternating galactosyluronic acid and rhamnosyl residues. Pectin compounds form the key binding material between plant cells. Hydrolysis of pectin compound is an important step in the enzymatic hydrolysis of citrus peel because it has high pectin content (>30%, Yapo *et al.*, 2007).

Pectate lyases play a major role in bacterial pathogenesis. After invading the host plant tissue, *Erwinia* produces a large amount of cell wall-degrading enzymes, generating typical soft rot symptoms. *Erwinia* bacteria secrete several isoenzymatic forms of pectate lyase that degrade pectin into unsaturated OG, the major virulence determinant of *Erwinia*, known to trigger plant defence responses (Ryan, 1988). Therefore, it has been shown that the expression of pectate lyase in potato enhanced resistance to *Erwinia* soft rot (Wegener, 2002). Different pectate lyases have been isolated from *Fusarium solani* f. sp. pisi (Gonzalez-Candelas and Kolattukudy, 1992; Guo *et al.*, 1995, 1996). These pectate lyases belong to polysaccharide lyase family 3 (http://www.cazy.org/fam/PL3.html). *Fusarium solani* f. sp. pisi, is a causative agent of root rot disease in pea (Funnell *et al.*, 2001) and chickpea plants (Bhatti and Kraft, 1992). Pectate lyases are also produced by other disease causing organisms like *Erwinia*, *Bacillus*, *Aspergillus* and many other plant pathogenic organisms. *Fusarium solani* f. sp. pisi produces at least four pectate lyases of which PelA and PelD are inducible and PelB and PelC are constitutively expressed (Rogers *et al.*, 2000). Therefore, in this study, PelB and PelD have been expressed in chloroplasts and used in enzyme cocktails for biomass hydrolysis or their role in enhanced resistance to *Erwinia* soft rot has been investigated.

Cellulosic biomass or lignocellulosic biomass is a heterogeneous complex of different polymers (Sticklen, 2008). Acid or alkaline pretreatment of wood biomass makes this substrate more accessible to enzymes and converts cellulosic polymers into fermentable sugars (Margeot *et al.*, 2009; Merino and Cherry, 2007; Wyman *et al.*, 2005). Likewise, citrus waste is rich in pectin, cellulose and hemicellulosic polysaccharides, which can be hydrolyzed into sugars and fermented into ethanol. Citrus-processing plants in Florida annually yield about 5 million tons of wet waste, which has the potential to produce 200 million gallons of ethanol (*http://*www.nps.ars.usda.gov). Upon complete hydrolysis by enzymes, citrus waste should yield fermentable hexose sugars; monosaccharides, including galacturonic acid, which can be fermented to ethanol and acetic acid by the recombinant bacterium *Escherichia coli* KO11 (Grohmann *et al.*, 1994).

The conversion of cellulosic biomass into fermentable sugars is a complex process and involves enzymatic hydrolysis using three major classes of enzymes including endoglucanase, exoglucanase and beta glucosidase. Especially the endoglucanases (EC No. 3.2.1.4) constitute an important enzyme group for hydrolysis. Endoglucanases catalyze endohydrolysis of 1,4-beta-D-glycosidic linkages in cellulose, cellulose derivatives (such as carboxy methyl cellulose and hydroxy ethyl cellulose), lichenin, beta-1,4 bonds in mixed beta-1,3 glucans such as cereal beta-D-glucans or xyloglucans and other plant materials containing cellulosic components. Several endoglucanases from various organisms including *Acidothermus cellulolyticus*, *Syncephalastrum racemosum*, *Thermobifida fusca*, *Trichoderma viride*, *Aspergillus niger*, *Bacillus polymyxa* and *Clostridium thermocellum* have been well characterized (Baird *et al.*, 1990; Hasper *et al.*, 2002; Irwin *et al.*, 1993; Kwon *et al.*, 1999; Ng and Zeikus, 1981; Wonganu *et al.*, 2008).

As the lignocellulosic wastes are composed of a complex of multiple intertwined polymers, simultaneous presence of multiple hydrolases that can increase the access of each other will be required to get efficient release of monomers. Thus, a mixture of enzymes like hemicellulases including xylanase, acetyl xylan esterase and ligninases,

lipases, pectate lyases may be required for efficient hydrolysis, depending upon the composition of cell walls as it varies depending on plant taxa, tissue, age and cell type (Sticklen, 2008). For example, in orange peel that has high pectin content requires high dosage of pectinase, whereas wood biomass requires high dosage of xylanase and its accessory enzymes like acetyl xylan esterase and ferulic acid esterase for efficient hydrolysis. Enzymes like expansins have been proposed to disrupt hydrogen bonding between cellulose microfibrils or between cellulose and other cell wall polysaccharides without having hydrolytic activity, and including this enzyme for biomass hydrolysis enhances the access of other enzymes for hydrolysis (Saloheimo *et al.*, 2002). In some biomass, such as citrus peel, a cutin layer is present in the epidermal layer and therefore hydrolysis of this polymer by cutinase is likely to enhance the access of hydrolases underlying carbohydrate polymers. All these enzymes are produced naturally by a range of microbial species including bacteria and fungi. Many cell wall-degrading enzymes have been isolated and characterized and many more are still not uncovered. Availabilty of genome sequences of *Trichoderma reesei* (Martinez *et al.*, 2008) and other organisms (Rubin, 2008) have increased inventory of enzymes for biomass utilization. Expression of all different classes of cell wall-degrading enzymes individually provides great opportunity for developing biomass-specific enzyme cocktails and no such plant-derived enzyme cocktail has been reported so far in the literature.

Production of cellulosic ethanol is currently limited by the lack of technology, infrastructure and high cost of enzymes. Because of their complex structure, lignocellulosic biomass degradation requires different classes of enzymes in large quantities to efficiently release fermentable sugars. Bioethanol process would require about 11 million filter paper units (FPU) of cellulase (19 kg, 42 lbs) to yield 84 gallons of ethanol (Himmel *et al.*, 1997, 1999) or 15–25 kg cellulase per ton of biomass (Carroll and Somerville, 2009; Taylor *et al.*, 2008). Moreover, because of the different polymer compositions, it is necessary to produce different classes of enzymes individually and then create cocktails for hydrolysis of different types of biomass. Therefore, the first challenge in lignocellulosic biotechnology is to develop an efficient enzyme production system for rapid and less expensive biomass depolymerization. Because of the high cost and limited capacity for producing these enzymes through fermentation, in planta expression of biomass-degrading enzymes should lower the cost of cellulosic ethanol.

Although several reports have investigated heterologous cellulase production in plants, most utilized the nuclear transformation technology (Dai *et al.*, 2000; Kawazu *et al.*, 1999; Taylor *et al.*, 2008; Ziegelhoffer *et al.*, 1999). So far, the beta 1,4 endoglucanase (E1), cellulases, xylanases, alpha glucosidase, amylases, mixed-linkage glucanases from a variety of bacteria and fungi have been investigated (Biswas *et al.*, 2006; Dai *et al.*, 2000; Kawazu *et al.*, 1999; Montalvo-Rodriguez *et al.*, 2000; Oraby *et al.*, 2007; Taylor *et al.*, 2008; Xu *et al.*, 2008; Ziegelhoffer *et al.*, 1999, 2001). None of these studies have used real lignocellulosic biomass as substrate or determined the combination and concentration of enzymes for the development of enzyme cocktails for biomass hydrolysis. Production of enzymes via plant nuclear transformation has a few limitations including lower levels of expression (with a few exceptions), gene silencing and position effect (Verma and Daniell, 2007). In contrast, plastid transformation results in high levels of expression, with minimal concerns of transgene silencing or position effect (Bally *et al.*, 2009; Daniell *et al.*, 2001; DeCosa *et al.*, 2001; Lee *et al.*, 2003; Singh *et al.*, 2008; Verma and Daniell, 2007). Compartmentalization of toxic proteins within chloroplasts protect transgenic plants from pleiotropic effects (Daniell *et al.*, 2001; Lee *et al.*, 2003). Most importantly, harvesting leaves before flowering offers nearly complete transgene containment, in addition to protection offered by maternal inheritance of transgenes, especially in tobacco (Daniell, 2007; Ruf *et al.*, 2007; Svab and Maliga, 2007). Therefore, bacterial genes have been expressed via the tobacco plastid genome for biofuel enzyme production (Gray *et al.*, 2008; Leelavathi *et al.*, 2003; Yu *et al.*, 2007). However, fungal genes have not yet been expressed in transgenic chloroplasts because of concerns of codon usage or appropriate post-translational modifications. Furthermore, efficacy of enzyme cocktails derived from plants for production of fermentable sugars from biomass has not yet been investigated (Taylor *et al.*, 2008).

In this study, we have used endoglucanase, exoglucanase or lipase from bacteria, pectate lyases, cutinase, endoglucanases, swollenin, xylanase, acetyl xylan esterase or beta glucosidase from fungi to create chloroplast vectors. A PCR-based method was used to clone open reading frames without introns (up to five) from *T. reesei* genomic DNA using An *et al.* (2007) protocol. Because chloroplast vectors function efficiently in *E. coli* (Brixey *et al.*, 1997), it was possible to express enzymes in both systems. Enzyme cocktails were used for biomass degradation to produce fermentable sugars and for direct comparison of

properties of enzymes produced via fermentation or in planta, using identical genes and regulatory sequences. Observed results indicate that plant-derived enzymes offer an inexpensive and efficient method to produce fermentable sugars from lignocellulosic biomass.

Results and discussion

Assembly of chloroplast expression constructs

For the integration of transgenes, transcriptionally active spacer region between the *trn*I and *trn*A genes was used (Figure 1a). This region allows highly efficient transgene integration and expression (Arlen *et al.*, 2008; Daniell *et al.*, 2009; DeCosa *et al.*, 2001; Koya *et al.*, 2005; Lelivelt *et al.*, 2005). PCR resulted in the amplification of various genes of interest (GOI) including endoglucanase (*cel*D), exoglucanase (*cel*O) from *C. thermocellum* genomic DNA, lipase (*lip*Y) from *Mycobacterium tuberculosis* genomic DNA, pectate lyases (*pel*A, *pel*B, *pel*D) and cutinase from *F. solani*. Because of the existence of introns, low copy number of genes, high complexity of the eukaryotic genome and multiple steps involved in cDNA preparation, it is very challenging to clone full-length cDNA from fungi. Using a PCR-based method (An *et al.*, 2007), coding sequences of GOI including endoglucanase (*egl*), swollenin (*swo*1 similar to expansins), xylanase (*xyn*2), acetyl xylan esterase (*axe*1) and beta glucosidase (*bgl*1) were cloned without introns (ranging from 1 to 5) from *T. reesei* genomic DNA. This method can be used to isolate any gene from genomic DNA of an organism whose genomic sequence is available. Tobacco chloroplast transformation vectors were made with each GOI (Figure 1b). All chloroplast vectors included the 16S-*trn*I/*trn*A-flanking sequences for homologous recombination into the inverted repeat regions of the chloroplast genome and the *aad*A gene conferring resistance to spectinomycin. The origin of replication, oriA exists inside the *trn*I-flanking region and might assist in replication of foreign vectors within chloroplasts (Daniell *et al.*, 1990), thereby increasing the chances of transgene integration and reach homoplasmy even in the first round of selection (Guda *et al.*, 2000). The *aad*A gene was driven by the constitutive rRNA operon promoter with GGAGG ribosome-binding site. The GOI was driven by the *psb*A promoter and 5′ UTR to achieve high levels of expression. The 3′ UTR located at the 3′ end of the GOI conferred transcript stability. The *aad*A gene conferring spectinomycin resistance was used for selection.

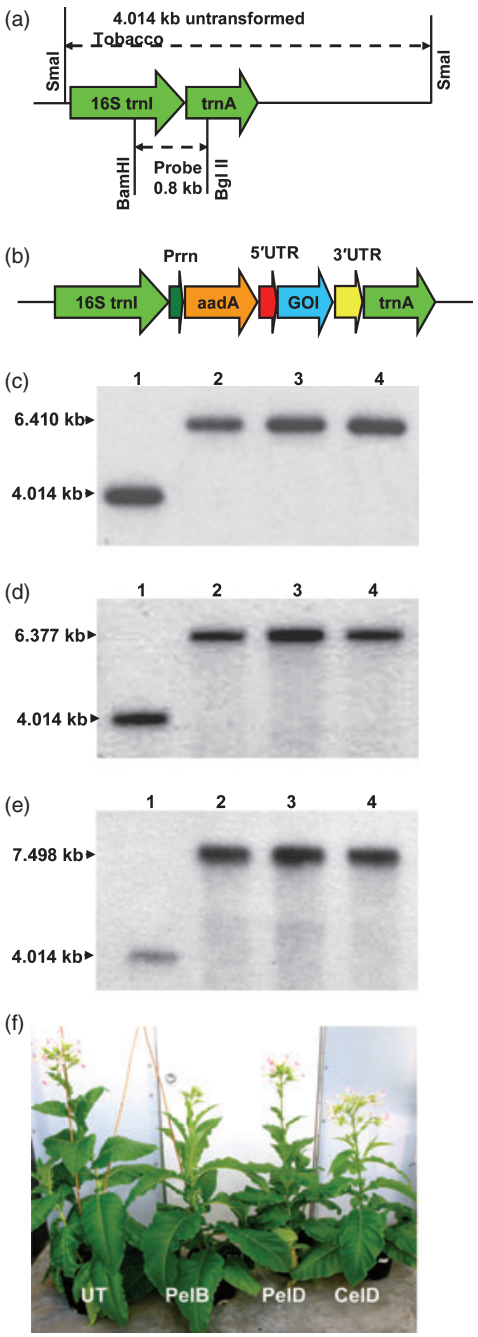

Figure 1 Regeneration and analysis of transplastomic lines (a) Schematic representation of the chloroplast 16S *trn*I/*trn*A region. Transgenes were inserted at the *trn*I/*trn*A spacer region in the tobacco chloroplast genome. (b) Schematic representation of the chloroplast transformation vectors. The gene of interest is *cel*D, *cel*O, *pel*A, *pel*B, *pel*D, cutinase, *lip*Y, *egl*, *swo*1, *xyn*2, *axe*1 or *bgl*1. Prrn, rRNA operon promoter; *aad*A, aminoglycoside 3′-adenylytransferase gene; 5′ UTR, promoter and 5′ untranslated region of *psb*A gene; 3′ UTR, 3′ untranslated region of *psb*A gene. (c) Evaluation of transgene integration and homoplasmy by Southern blot of *pel*B, (d) *pel*D and (e) *cel*D transplastomic Petite Havana lines hybridized with the flanking sequence probe (1, untransformed; 2–4, transplastomic lines). (f) Phenotypes of untransformed (UT) and transplastomic lines (Petite Havana) grown in green house showing normal growth.

Generation and characterization of transplastomic tobacco expressing pectate lyases (PelB & PelD) and endoglucanase (CelD)

Transplastomic tobacco plants of experimental Petite Havana cultivar were obtained as described previously (Daniell *et al.*, 2005; Verma *et al.*, 2008). Southern blot analysis was performed to confirm site-specific integration of the pLD-pelB, pLD-pelD and pLD-celD cassettes into the chloroplast genome and to determine homoplasmy. Digestion of total plant DNA with *Sma*I from untransformed and transplastomic lines generated a 4.014 kb fragment untransformed (UT) or 6.410 kb in *pel*B, 6.377 kb in *pel*D or 7.498 kb fragment in *cel*D when hybridized with the [^{32}P]-labelled *trn*I-*trn*A probe, confirming site-specific integration of the transgenes into the spacer region between the *trn*I and *trn*A genes (Figure 1c–e). Furthermore, the absence of a 4.014 kb fragment in the transplastomic lines confirmed that homoplasmy was achieved (within the levels of detection). Transplastomic lines showed normal phenotype when compared to untransformed plants and were fertile (produced flowers, seeds Figure 1f).

Immunoblots with antibodies raised against PelA and inhibition of pectate lyase activity in the presence of PelA antibody showed that PelB and PelD are immunologically related to PelA (Guo *et al.*, 1995, 1996). Therefore, PelA antibody was used to detect the expression of PelB and PelD, although their affinity was variable in transplastomic lines. All transplastomic lines showed similar expression levels of PelB or PelD at different times of harvest, even though both transgenes were regulated by light (Figure 2a,b). This may be because of variable affinity between antigen epitopes of PelB, PelD and PelA antibody. Enzyme concentration slightly changed with leaf age and decreased in older leaves (Figure 2a,b). Western blots for expression of PelB and PelD from *E. coli* were performed using the His-tag antibody because PelA antibody cross-reacted with too many proteins in *E. coli* cell extract, but not with any other protein in plant extract. Western blots show that PelB and PelD are expressed well in *E. coli* (Figure 2c). However, the His-tag antibody could not detect the chloroplast-derived PelB and PelD (Figure 2c). This could be because of the difference in folding of these proteins after formation of disulphide bonds in chloroplasts, making the His-tag inaccessible to the antibody. CelD western blots could not be performed because of nonavailability of this antibody.

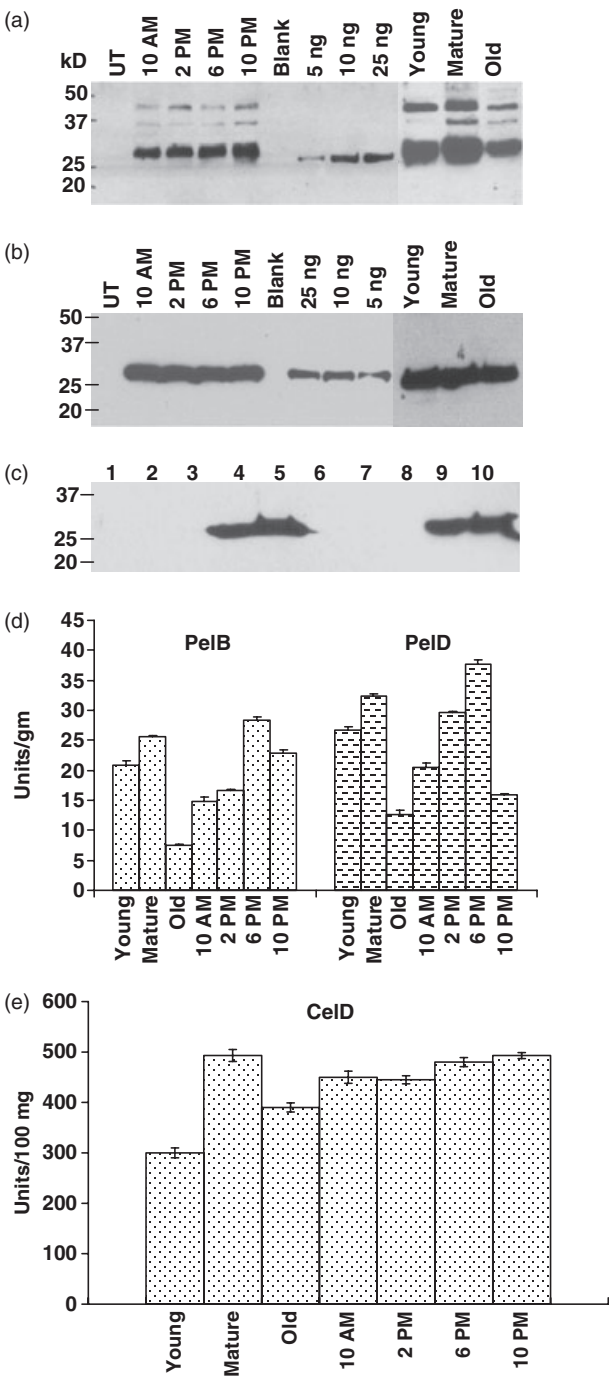

Figure 2 Western blot analysis and quantification of transplastomic lines. Western blot of transplastomic lines expressing (a) PelB or (b) PelD. UT: untransformed, mature leaves harvested at 10 AM, 2, 6 and 10 PM; 5, 10 and 25 ng: PelA purified protein, young, mature and old leaves. (c) Western blot analysis of PelB and PelD with His-tag antibody. Lane 1, protein marker, lane 2, untransformed plant extract; lane 3, PelD plant extract; lanes 4 and 5, PelD *Escherichia coli*; lane 6, untransformed *E. coli*; lane 7, PelB plant extract; lane 8, blank; lanes 9 and 10, PelB *E. coli*. Enzyme units of PelB and PelD (d) or CelD (e) from 1 g or 100 mg leaf of different age or harvesting time.

Quantification of pectate lyases (PelB, PelD) and endoglucanase (CelD) at different harvesting time and leaf age

The activity of the enzyme varied significantly depending on the developmental stage and time of leaf harvest. Maximum enzyme activity was observed in mature leaves of PelB, PelD and CelD, with reduced activity in older leaves (Figure 2d,e). Mature leaves harvested at 6 PM showed maximum activity in both PelB and PelD, whereas CelD showed maximum activity at 10 PM (Figure 2d,e). This may be because of increased stability of endoglucanase against proteases in plant extracts. Activity of cpCelD did not significantly decrease in plant crude extracts stored at room temperature, for more than 30 days (data not shown).

CelD enzyme activity was calculated using the dinitrosalicylic acid (DNS) reagent (Miller, 1959) according to the IUPAC protocol (Ghose, 1987). The specific activity of cpCelD using 2% carboxymethyl cellulose (CMC) substrate was 493 units/mg total soluble protein (TSP) or 100 mg leaf tissue, in crude extracts prepared from mature leaves harvested at 10 PM. Using the glucose hexokinase assay, which is highly specific for glucose, the specific activity was 4.5 units/mg TSP and 6.28 units/mg TSP, when 5% Avicel and Sigmacell solution, respectively, was used as substrate (at pH 6.0, 60 °C).

CelD is known to have activity on microcrystalline substrate like Avicel and BMCC (Carrard *et al.*, 2000; Fukumura *et al.*, 1997; Kataeva *et al.*, 1997). Endoglucanases randomly break down the β (1 → 4) glycosidic bonds existing between glucose molecules in cellulose. Some endoglucanases, although may not be active on cellobiose, hydrolyze microcrystalline substrate and release not only various lengths of cello-oligosaccharides but also individual glucose molecules. For example, CelT of *C. thermocellum* is also an endoglucanase without carbohydrate-binding domain (like CelD), hydrolyzes Avicel and releases individual molecules of glucose (Kurokawa *et al.*, 2002). We did not find any detectable endogenous beta glucosidase activity in untransformed plant extracts, under our experimental conditions. Therefore, based on appropriate negative controls, authors are confident that the glucose released is indeed from the activity of CelD in transplastomic crude extracts.

Figures 2d,e show that approximately 26, 32 and 4930 units of PelB, PelD and CelD were obtained per gram fresh weight of mature leaves harvested at 6 or 10 PM. Thus, 2048, 2679 and 447, 938 units of PelB, PelD and CelD can be harvested from each tobacco plant (experimental cultivar, Petite Havana). With 8000 tobacco plants grown in one acre of land, 16, 21 and 3584 million units of PelB, PelD or CelD can be obtained per single cutting (Table 1). Based on three cuttings of tobacco in 1 year, up to 49, 64 and 10 751 million units of PelB, PelD or CelD can be harvested each year. The commercial cultivar yields 40 metric tons biomass of fresh leaves as opposed to 2.2 tons in experimental cultivar Petite Havana. Therefore, the commercial cultivar is expected to give 18-fold higher yields than the experimental cultivar.

Table 1 Enzyme yield in transplastomic tobacco plants

Enzyme	Leaf age	No of leaves/ plant	Avg. Wt (g)/ leaf	Units/g in fresh leaf	Units		Whole plant yield, %	Units(millions)/ acre/cutting	Units(millions)/ acre/year
					Per leaf	Per age group			
PelB	Young	3.5	2.5	20.82	52.05	182.18	8.89	16.39	49.17
	Mature	8.2	8.0	25.56	204.48	1676.74	81.84		
	Old	4.5	5.6	7.54	42.22	190.00	9.27		
PelD	Young	3.5	2.5	26.65	66.63	233.19	8.70	21.43	64.30
	Mature	8.2	8.0	32.44	259.52	2128.06	79.43		
	Old	4.5	5.6	12.62	70.67	318.02	11.87		
CelD	Young	3.5	2.5	3000.00	7500.00	26 250.00	5.86	3583.50	10751.00
	Mature	8.2	8.0	4930.00	39 440.00	323 408.00	72.20		
	Old	4.5	5.6	3900.00	21 840.00	98 280.00	21.94		

One unit of PelB and PelD enzyme is defined as the amount of enzyme that forms 1 μmol of unsaturated soluble oligogalacturonates per min with a molar extinction coefficient of 4600 per μmol/cm in a 2.5-mL reaction containing 2.5 mg/mL polygalacturonic acid.

One unit of CelD enzyme is defined as the amount of enzyme that released 1 μmole glucose equivalents per minute in a 1-mL reaction containing 2% CMC.

Effect of pH & temperature on pectate lyases (PelB & PelD) and endoglucanase (CelD) enzyme activity

The goal of this study is to use crude extracts and not purified enzymes to achieve low cost production. Therefore, the kinetic data provided in this manuscript are only operational parameters and such parameters are needed for development of enzyme cocktails (range of pH, functional temperature, etc.) and in the context of the applications of the results for production of soluble sugars from agricultural wastes. Further characterization of the expressed enzymes is not particularly useful for this application. Several laboratories have reported characterization of enzyme activities using crude plant extracts (Bae *et al.*, 2008; Dai *et al.*, 2000; Gray *et al.*, 2008; Jin *et al.*, 2003; Leelavathi *et al.*, 2003; Montalvo-Rodriguez *et al.*, 2000; Oraby *et al.*, 2007; Sun *et al.*, 2007; Yu *et al.*, 2007; Zeigler *et al.*, 2000; Ziegelhoffer *et al.*, 1999; Ziegelhoffer *et al.*, 2009).

Both plant and *E. coli* extracts showed optimal pectate lyase activity at 2.5 mg/mL polygalacturonic acid (PGA, Figure 3a). Therefore, all enzyme characterization studies were performed at this substrate concentration. Kinetic studies carried out by using 4 μg of TSP, with increasing concentration of PGA (0–2.5 mg), under standard assay conditions gave K_m values of 0.39 and 1.19 μg/mL in chloroplast (cp) and *E. coli* (r) PelB, respectively, whereas values for chloroplast and *E. coli* PelD were 0.50 and 1.29 μg/mL, respectively. The V_{max} values obtained were 2.75, 3.19, 2.75 and 3.14 units/mg for cpPelB, rPelB, cpPelD and rPelD, respectively (Figure 3a).

The crude extract (4–5 μg TSP) from plant or *E. coli* was used to study the effect of pH and temperature on the activity of enzymes. The optimal pH for the *E. coli*-derived pectate lyase in the presence or absence of 1 mM $CaCl_2$ under the standard assay conditions was 8.0 whereas plant-derived pectate lyases showed a pH optimum of 6.0 in the presence of $CaCl_2$ or 8.0 in the absence of Ca^{2+} ions (Figure 3b,c). The pH stability curve showed that the pectate lyase activity of chloroplast and *E. coli*-derived enzyme in the absence of $CaCl_2$ remained over 79% and 71%, respectively, in the buffers ranging from pH 6.0 to pH 9 (Figure 3b). However, increasing the pH to 10 resulted in decline of activity of chloroplast and *E. coli*-derived enzyme to about 38% and 33%, respectively. In the presence of $CaCl_2$, pectate lyase B and D (both chloroplast and *E. coli* derived) retained over 76% and 67% activity, respectively, in the buffers ranging from pH 6.0 to pH 9 and retained 29%–42% activity at pH 10 (Figure 3c). The pH stability curve for endoglucanase (CelD) showed that the endoglucanase activity of chloroplast and *E. coli* derived enzyme remained over 65% and 35%, respectively, in the buffers ranging from pH 4.5 to pH 7.5 (Figure 3f). However, at pH 9 complete loss in enzyme activity was observed irrespective of source of enzyme (Figure 3f).

The optimal temperature for the *E. coli* and chloroplast-derived pectate lyase in the presence or absence of 1 mM $CaCl_2$ under the standard assay conditions was 40 °C (Figure 3d,e). The chloroplast-derived pectate lyases retained 65%–76% activity at 70 °C temperature whereas *E. coli*-derived pectate lyase retained only 25%–34% activity (Figure 3d,e). The temperature stability curve for endoglucanase (CelD) showed that the enzyme activity increased with increasing temperature up to 70 °C in both *E. coli* and chloroplast-derived endoglucanase. Further increase in temperature resulted in rapid decline of enzyme activity (Figure 3g). These data show that the chloroplast-derived pectate lyases and endoglucanases are reasonably stable up to 70 °C. Untransformed *E. coli* and leaf crude extracts did not yield any detectable amount of unsaturated galacturonic acid/glucose equivalents under standard assay conditions (data not shown).

Clostridium thermocellum CelD is structurally known to have affinity for $CaCl_2$ ions and it also provides thermostability (Chauvaux *et al.*, 1990). Even though 10 mM $CaCl_2$ increased CelD activity in 2% CMC to twofold in *E. coli* crude extract, this was not apparent in chloroplast CelD crude extract during initial period of incubation. This may be because of the optimum concentration of calcium ion present in plant cells. However, $CaCl_2$ with 20 μg BSA yielded fivefold increased activity at the end of 36 h incubation for cpCelD crude extract (Figure 3h). Even though Ca^{2+} ions are required for pectate lyase activity it is unclear whether it binds to the enzyme (Crawford and Kolattukudy, 1987). However, Yoder *et al.* (1993) found a putative binding site for Ca^{2+} on the outside of parallel β sheet of PelC pectate lyase of *Erwinia*. Ca^{2+} ions also play role in the cross-linking of pectins during plant cell wall development and organization of plant cell wall polysaccharides (Carpita and Gibeaut, 1993; Wellner *et al.*, 1998). Ca^{2+} ions also influenced the enzyme stability of PelB and PeD at higher temperature (40–55 °C) when compared to enzymes expressed in *E. coli* (Figure 3d,e) or purified enzymes which had optimum temperature of 30 °C except for PelC which had 55 °C as optimum temperature (Guo *et al.*, 1995). These differences in enzyme properties from two different hosts may be due to their folding. This possibility is supported by the observation

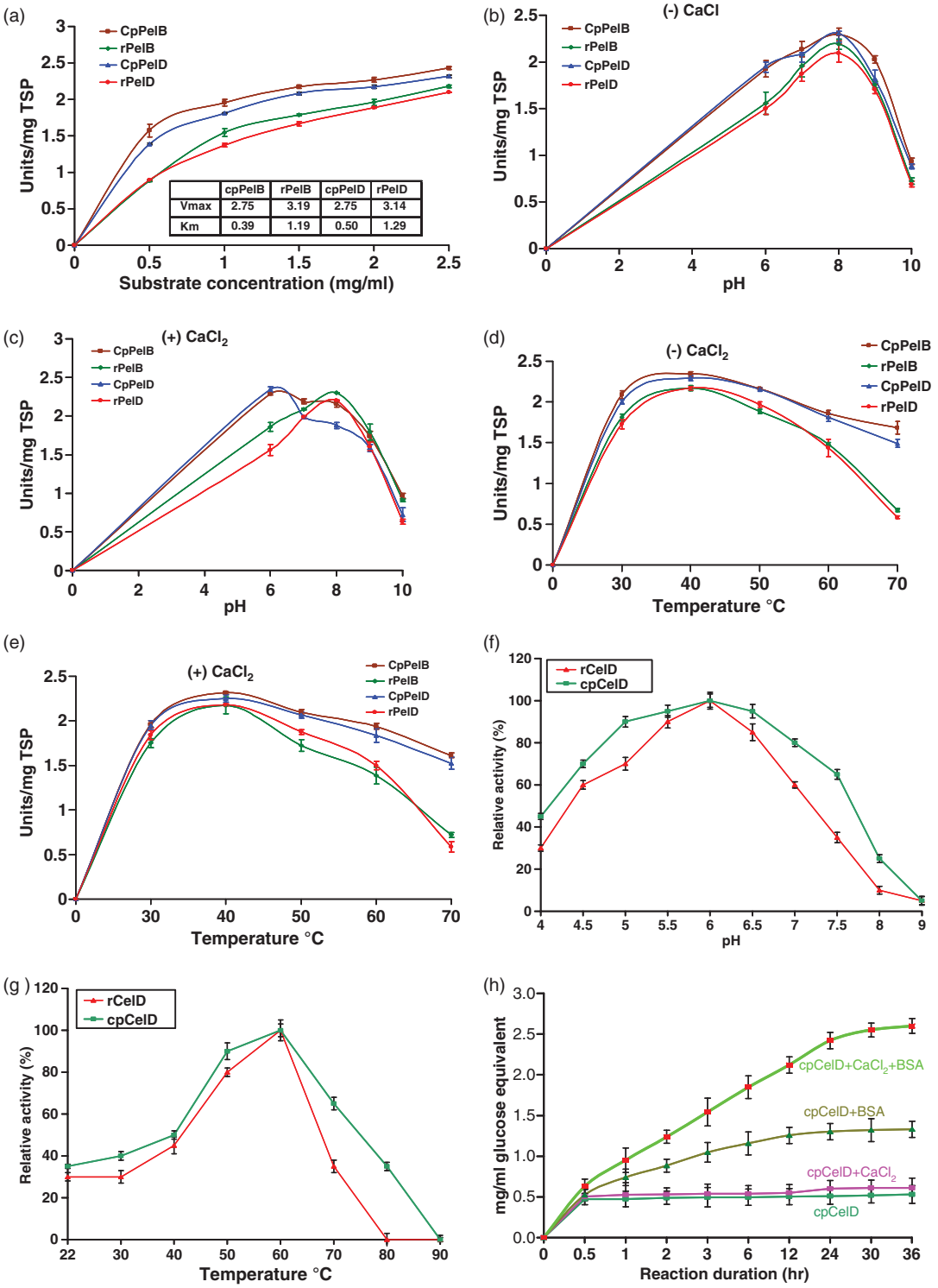

Figure 3 Effect of substrate, pH, temperature and cofactors on cpPelB, rPelB, cpPelD, rPelD, rCelD and cpCelD enzyme activity. (a) Effect of increasing PGA concentration on pectate lyases activity. (b) Effect of pH on pectate lyases activity in the absence of $CaCl_2$ and (c) in the presence of $CaCl_2$. (d) Effect of temperature (30–70 °C) on enzyme activity at pH 8.0 in the absence of $CaCl_2$ and (e) in the presence of $CaCl_2$. (f) Optimization of pH and (g) effect of increasing temperature on cpCelD and rCelD enzyme activity. (h) Enhancement of cpCelD (25 μg total soluble protein/mL reaction) activity using 10 mM $CaCl_2$ and 20 μg/mL BSA individually or in combination with 50 mM sodium acetate during the prolonged enzymatic hydrolysis. The hydrolysis was carried out up to 36 h at 60 °C, pH 6.0 in the presence of CMC (2%). Untransformed *Escherichia coli* and leaf crude extracts did not yield any detectable level of unsaturated galacturonic acid or reducing sugar under these assay conditions.

that it was possible to detect the *E. coli* enzyme with HIS-tag antibody but not the chloroplast enzyme (Figure 2c). It is well known that foreign proteins form disulphide bonds in chloroplasts (Arlen *et al.*, 2007; Bally *et al.*, 2008; Ruhlman *et al.*, 2007) but not in *E. coli* when expressed in the cytoplasm. Both PelB and PelD enzymes have even number (12 or 14) cysteines that could form disulphide bonds (Guo *et al.*, 1995).

Escherichia coli vs. Chloroplast CelD, PelB & PelD

Escherichia coli crude extract containing CelD enzyme showed decrease in enzyme activity when the reaction mixture contained more than 10 μg TSP, where as plant crude extract containing CelD released more reducing sugar with increasing protein concentration (Figure 4a).

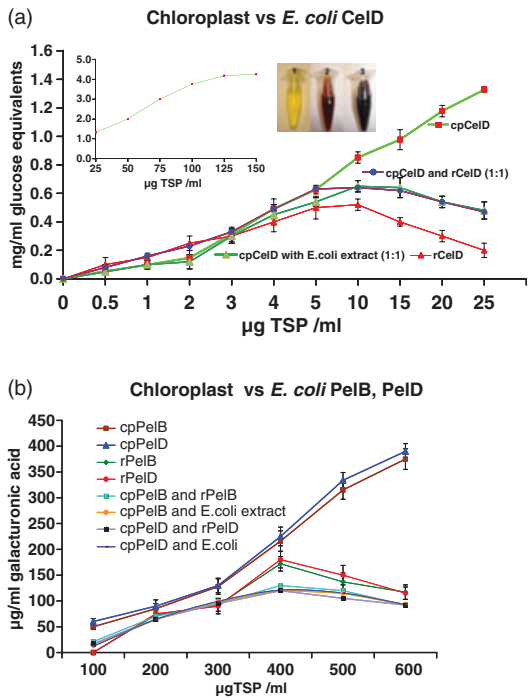

Figure 4 *Escherichia coli* vs. chloroplast-derived enzymes at different protein concentrations of crude extracts. (a) Enzyme kinetics of cpCelD and rCelD using carboxymethyl cellulose (2%) substrate. The reaction mixture contained increasing concentration of cpCelD and rCelD total soluble protein (TSP) (μg/mL) with 10 mM $CaCl_2$ in 50 mM sodium acetate buffer, pH 6.0. Enzyme hydrolysis was carried out for 30 min at 60 °C. Figure inset shows enzyme kinetics saturation point for cpCelD TSP amount (μg/mL) towards CMC (2%). Eppendorf tubes with reaction mixture shown in inset represents, 1 untransformed plant, 2 and 3 rCelD and cpCelD 10 μg TSP. (b) Effect of cpPelB, cpPelD, rPelB, and rPelD on hydrolysis of 5.0 mg/mL sodium polygalacturonate substrate. The reaction mixture contained increasing concentration of cpPelB, cpPelD, rPelB and rPelD (μg/mL) in 20 mM Tris–HCl buffer (pH 8.0). Enzyme hydrolysis was carried out for 2 h at 40 °C on rotary shaker at 150 r.p.m.

Chloroplast expressed CelD activity was saturated (in 2% CMC) at 150 μg TSP (Figure 4a inset) and there was no decrease in chloroplast CelD enzyme activity even up to 500 μg TSP as determined by end point assay. This finding is potentially of high practical significance because use of crude extracts eliminates the need for expensive purification steps. Large amounts of crude plant enzymes can be utilized in the cocktail as shown below without causing detrimental effect on enzyme activities, hydrolysis or yield of end products.

Crude plant extracts containing cpCelD, cpPelB or cpPelD can be directly used for biomass degradation without any need for purification whereas *E. coli* extracts probably contain endoglucanase inhibitors. At higher protein concentrations, *E. coli* expressing rCelD, rPelB or rPelD showed reduced pectate lyase or endoglucanase activity and therefore prohibits high protein loading for higher hydrolysis whereas cpCelD, cpPelB or cpPelD continued to increase activity even up to 600 μg TSP. There may be inhibitors of enzyme activities in *E. coli* extracts, which are not present in plant crude extracts. Addition of *E. coli* crude cell extract from untransformed control or cells expressing CelD or pectate lyase inhibited CelD or pectate lyase activities in plant extracts (Figure 4a,b). Crude extracts were used for assays without any dilution or concentration in both *E. coli* and tobacco. Therefore, molar ratio of protein and inhibitor was not changed under our experimental conditions. Inhibition observed in *E. coli* at higher protein concentrations is not because of the expressed enzymes or products formed. Furthermore, product inhibition is not a possible explanation because higher products are formed in tobacco extracts than in *E. coli*.

Resistance of transplastomic pectate lyase plants to *Erwinia carotovora*

The ability of pectate lyase to trigger plant defence responses was studied by investigating enhanced resistance to *Erwinia* soft rot either by using syringe or sand paper method. One day after inoculation with *Erwinia*, the first signs of damage were observed on leaves of untransformed plants in the region of the inoculated surface. On the third day, virtually all inoculated untransformed leaf surfaces underwent necrosis, whereas in leaves of transgenic plants, no damage zone was observed. Inoculation of potted plants with *E. carotovora* using a sandpaper technique and needle/syringe method resulted in areas of necrosis surrounding the point of inoculation in

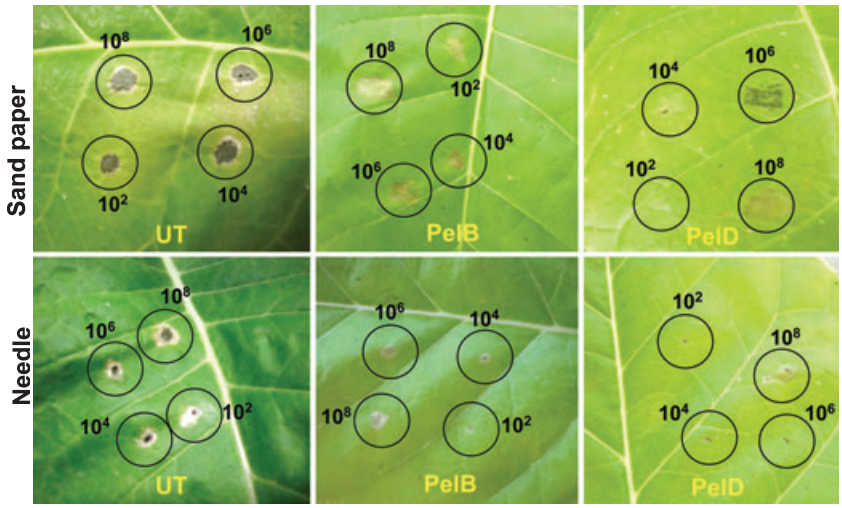

Figure 5 In planta bioassays 5- to 7-mm areas of untransformed, PelB and PelD transplastomic tobacco cv Petite Havana leaves were scraped with fine-grain sandpaper. Twenty microlitres of 10^8, 10^6, 10^4 and 10^2 cells from an overnight culture of *Erwinia carotovora* were inoculated to each prepared area. Photos were taken 5 days after inoculation.

untransformed control for all cell densities, whereas transplastomic PelB and PelD mature leaves showed no areas of necrosis (Figure 5). Even inoculation of 10^8 cells resulted in no necrosis in mature transplastomic leaves. However, untransformed plants inoculated with 10^2 cells displayed necrosis. Similar results were obtained with bacteria inoculated using a syringe (Figure 5). Transplastomic mature leaves injected with *E. carotovora* showed a mild discoloration at the site of inoculation of 10^8 cells. These results support the hypothesis that expression of pectate lyase induces plant defence responses.

Evaluation of enzyme activity for use in enzyme cocktail

Transplastomic lines were also generated expressing other biomass-degrading enzymes for use in enzyme cocktails for hydrolysis of different lignocellulosic biomass. To demonstrate that individual enzymes used in the cocktails are indeed active, crude extracts prepared from both *E. coli* and transplastomic plants were independently evaluated for enzyme activity using either model substrate (based on published data) or natural substrates (Table 2). As reported

Table 2 Enzyme activity of cell wall-degrading enzymes expressed in *Escherichia coli* and chloroplast

Enzyme	pH	°C	Substrate	Enzyme activity (units/mg) in crude total soluble protein		Activity of transplastomic over *E. coli* (fold)
				E. coli	Transplastomic	
CelD	5.2	60	CMC (2%)	349 ± 36	493 (±21)	1.41
EG1	5.2	50	CMC (2%)	28 ± 7	339 (±12)	12.10
CelO	5.2	60	β-D-glucan (1%)	18 ± 2	442 (±19)	24.55
Bgl1	5.2	50	p-Nitrophenyl-β-D-glucopyranoside (4 mM)	2 ± 0.02	14 (±2)	7.0
Xyn2	5.2	50	Oat spelt xylan (1%)	89 ± 3	421 ± 9	4.73
PelB	6 and 8	40	Polygalacturonic acid (0.25%)	2.17 ± 0.2	2.42 ± 0.1	1.12
PelD	6 and 8	40	Polygalacturonic acid (0.25%)	2.09 ± 0.3	2.31 ± 0.4	1.10
PelA	6 and 8	40	Polygalacturonic acid (0.25%)	2.50 ± 0.5	2.81 ± 0.9	1.12
Cutinase	8.0	30	p-Nitrophenyl butyrate (0.03%)	24 ± 4	15 ± 4	<0.625
SwoI	Swelling of cotton fibre was observed with *E. coli* and chloroplast-derived crude extract as described earlier (Saloheimo *et al.*, 2002).					
Axe1	Color change with *E. coli* enzyme extract was observed using 1 mM αnaphthyl acetate as described earlier (Poutanen and Sundberg, 1988)					

Enzyme assays were performed in triplicates and standard errors were calculated. Untransformed tobacco leaves and *E. coli* did not show detectable level of hydrolysis of any of the above substrates under conditions described in the materials and method.

above for pectate lyases or endoglucanase, enzyme activities should be measured at different developmental stages or leaves harvested at different times of the day and quantified using appropriate standards. As transplastomic plants expressing other enzymes were in different stages of development, we quantified enzyme activity with the available material. Chloroplast-derived pectate lyases and endoglucanase (CelD) had 1.1–1.41-fold higher activity when compared to *E. coli* crude extract. However, another endoglucanase (Egl) had 12.1-fold higher activity in plant extracts than *E. coli*. Maximum difference (24.55) in enzyme activity between *E. coli* and chloroplast-derived crude extract was observed in exoglucanase (CelO). Chloroplast-derived beta-glucosidase and xylanase also had several fold higher activity than *E. coli*-derived crude extract. Enzymes expressed in transplastomic tobacco chloroplasts performed better than those expressed in *E. coli*. This could be because of several reasons including absence of disulphide bond and improper folding, formation of inclusion bodies (e.g., EG III, Okada *et al.*, 1998; Sandgren *et al.*, 2001) or certain unknown inhibitory substances present in the *E. coli* crude extract, as repeatedly observed in this study.

The primary goal of our investigations was to use low cost crude extracts from *E. coli* or transplastomic plants. Therefore, we did not attempt any purification of enzymes. Pectate lyases are known to act synergistically in a variety of environment during degradation of its specific pectic compounds. Some of the reported pectate lyases also have activity on methylated pectin (Bartling *et al.*, 1995; Guo *et al.*, 1996). Therefore we used all three pectate lyases in the enzyme cocktails. Enzyme cocktails were prepared using crude soluble protein extracts from *E. coli* cultures or from transplastomic tobacco leaves, expressing the cell wall-degrading enzymes.

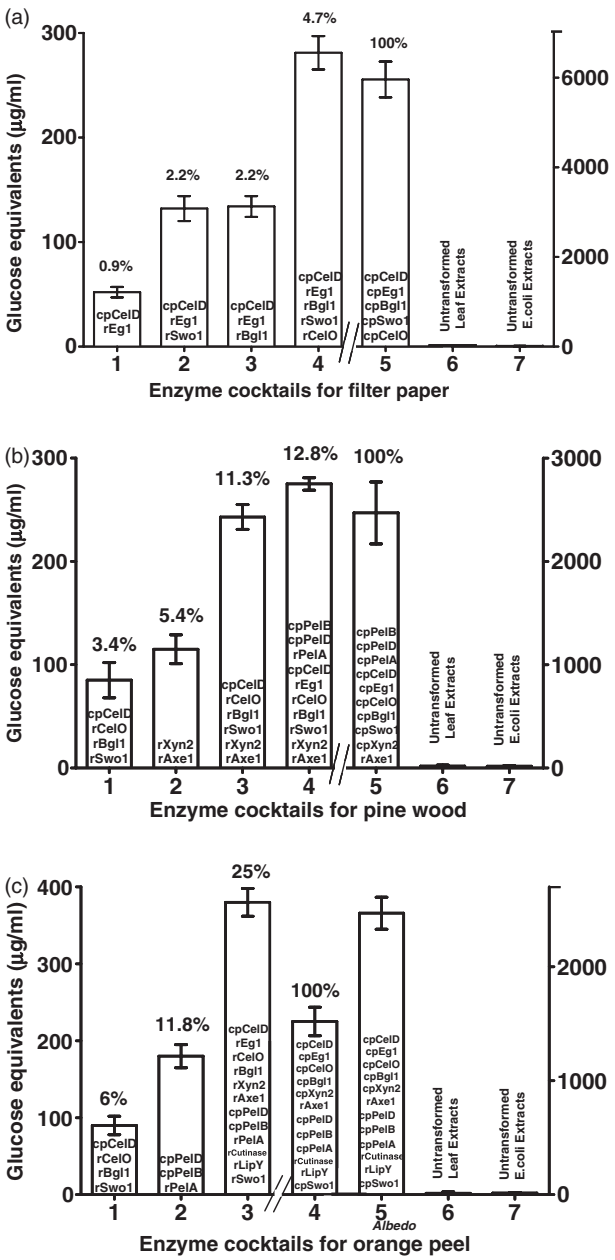

Figure 6 Enzyme cocktails for filter paper, pine wood and citrus peel. (a) Enzyme cocktail activity on Whatman No. 1 filter paper (50 mg/mL). (b) Hydrolysis of pine wood sample (200 mg/5 mL reaction). (c) Hydrolysis of Valencia orange peel and albedo portion (200 mg/5 mL reaction).

Enzyme cocktail for hydrolysis of filter paper

Before evaluation of enzyme cocktails, activity of each enzyme was tested independently with an appropriate substrate and enzyme units were calculated (Table 2). Chloroplast or *E. coli* expressed endoglucanase (cpCelD or rEg1) alone did not release any detectable glucose from filter paper but when mixed together up to 0.9% of total hydrolysis was observed (Figure 6a, bar 1). The synergistic activity was further enhanced up to 2.2% when the endoglucanases (cpCelD and rEg1) were mixed with swollenin or beta-glucosidase (Figure 6a, bars 2,3). The increase in hydrolysis with swollenin (expansin like) could be because of the loosening or disruption of the packaging of the plant cell wall and polysaccharides. Similar increase in the yield of sugar was observed with *Bacillus subtilis* expansin when filter paper was incubated with a mixture of expansin and low dosage of cellulase (Kim *et al.*, 2009). Addition of exoglucanase to this cocktail doubled the hydrolysis of filter paper (Figure 6a, bar 4). Observed synergism is probably because of the exo-mode of action of

cellobiohydrolase (Zverlov *et al.*, 2002) from reducing ends that were formed by random cuts in cellulose chains through endoglucanases, along with the action of swollenin and beta-glucosidase. It could also be because of the increased activity of exoglucanase in the presence of beta glucosidase, which reduces cellobiose, an exoglucanase inhibitor (Gupta and Lee, 2009). When we used the same cocktail but comprising of chloroplast expressed enzymes, there was 21.3-fold increase in hydrolysis releasing maximum amount of reducing sugars (Figure 6a, bar 5). Addition of leaf extract from untransformed plants and *E. coli* to filter paper didn't yield any detectable glucose (Figure 6a, bars 6,7). Although there are reports on synergistic effect of cellulase on substrates like Avicel, cotton fibres and filter paper (Gusakov *et al.*, 2007; Irwin *et al.*, 1993; Zhou and Ingram, 2000), they used purified recombinant enzymes expressed in bacteria or fungi but not crude extracts used in our study.

Enzyme cocktail for hydrolysis of pine wood

The enzyme cocktail mentioned in Figure 6a bar 4 for filter paper (except rEg1) resulted in 3.4% of total hydrolysis after 36 h (Figure 6b, bar 1) when tested on pine wood. An enzyme cocktail of endoxylanase and acetyl xylan esterase showed 5.4% of total hydrolysis (Figure 6b, bar 2). Similar interaction between xylanase and acetyl xylan esterase was noticed, confirming the strong synergistic relationship between these two enzymes (Kosugi *et al.*, 2002; Selig *et al.*, 2008). When the two cocktails (bar 1 and 2) were combined together, the hydrolysis increased up to 11.3% (Figure 6b, bar 3). Xylose removal probably enhanced cellulose accessibility and thus result in greater release of glucose. Supplementation of cellulase with xylanase enhanced glucose release from poplar pretreated solids and corn cell wall (Kumar and Wyman, 2009; Murashima *et al.*, 2003). Pectin is the major structural component of plant cell wall of woody plants including pine trees (Hafren *et al.*, 2000) along with cellulose and hemicellulose. Pectin is located mainly in the middle lamella and primary cell wall and functions as a matrix anchoring the cellulose and hemicellulose fibres (Carpita and Gibeaut, 1993). Therefore hydrolysis of pectin should result in loosening of cellulose and hemicellulosic fibres, resulting in enhanced glucose release by cellulases and hemicellulases. When pine wood substrate was first treated with pectate lyases, followed by the addition of the enzyme cocktail in bar 3, the overall hydrolysis was further enhanced up to 12.8% after 36 h incubation (Figure 6b, bar 4). When we used same cocktail but comprising of chloroplast expressed enzymes (except rAxe1), there was 7.8-fold increase in hydrolysis releasing maximum amount of reducing sugars (Figure 6b bar 5). Addition of leaf extract from untransformed plants and *E. coli* to pine wood did not yield any detectable glucose (Figure 6b, bars 6,7).

Enzyme cocktail for hydrolysis of citrus waste

The enzyme cocktail of endoglucanase (cpCelD), exoglucanase, swollenin and beta-glucosidase released up to 6% of total hydrolysis with citrus peel (Figure 6c, bar 1). When citrus peel was treated with pectate lyases (cpPelB, cpPelD and rPelA), hydrolysis was doubled (Figure 6c, bar 2) because of high pectin content (23%) in citrus peel (Yapo *et al.*, 2007). Addition of endoxylanase, acetyl xylan esterase, cutinase and lipase to the both these cocktails released 2.1-fold more glucose equivalents (Figure 6c, bar 3). Enzymes like cutinase and lipase may have hydrolyzed oil bodies present in the citrus peel, providing greater access to endoglucanase, endoxylanase and pectate lyases for efficient hydrolysis of citrus peel. Using the same cocktail from chloroplast expressed enzymes (except rCutinase, rLipY and rAxe1) resulted in 1520 and 2470 μg/mL (Figure 6c, bars 4,5) glucose equivalents from 200 mg of ground citrus peel and albedo portion of citrus peel, respectively, after 36 h incubation period. Addition of leaf extract from untransformed plants and *E. coli* to citrus waste didn't yield any detectable glucose (Figure 6c, bars 6,7). High amount of glucose released in albedo portion could be because of high content of cellulose and lack of oil bodies. Addition of other enzyme classes including the accessory enzymes produced in chloroplasts should further enhance yield of glucose in a cost effective manner.

Conclusions

Concerns over finite petroleum reserve require development of alternative energy resources. Lower emission of green house gases from alternative energy resources is also highly desirable. The fact that corn ethanol produces more green house gas emissions than gasoline and that cellulosic ethanol from nonfood crops produces less green house gas emissions than electricity or hydrogen, highly favours production of ethanol from cellulosic biomass. However, biofuel production from lignocellulosic materials is a challenging problem because of the multifaceted nature of raw materials and lack of technology to efficiently

and economically release fermentable sugars from the complex multi-polymeric raw materials. The high costs of enzyme production and the tremendous amount of enzymes needed to hydrolyse pretreated biomass are often considered as key obstacles in the commercial lignocellulosic ethanol industry (Margeot *et al.*, 2009). Therefore, in this study, we have used coding sequences from bacterial or fungal genomes to create chloroplast vectors. A PCR-based method was used to clone ORFs without introns from fungal genomic DNA. *Escherichia coli* expression system was used to evaluate functionality of each enzyme independently or their efficacy in enzyme cocktails before creating transgenic lines. The phenotype of homoplasmic transplastomic lines was normal and produced flowers and seeds. Based on three cuttings of tobacco in 1 year, 49, 64 and 10, 751 million units of pectate lyase and endoglucanase activity can be obtained each year in an experimental cultivar. Commercial cultivars yield 40 metric tons biomass of fresh leaves as opposed to 2.2 tons in experimental cultivar Petite Havana. Therefore, the commercial cultivar is expected to give 18-fold higher yields than the experimental cultivar. Because most enzymes for hydrolysis of plant biomass are active at higher temperatures, it is feasible to harvest leaves and sun dry them, as reported previously for chloroplast-derived xylanase (Leelavathi *et al.*, 2003). Moreover, in our study, activity of cpCelD did not decrease significantly in plant crude extracts stored at room temperature for more than 30 days. This is the first study which directly compared properties of enzymes produced in *E. coli* or in planta using identical genes and regulatory sequences. Chloroplast-derived enzymes showed higher temperature stability, broad pH optima and higher enzyme activity with increasing protein concentration than enzymes expressed in *E. coli* facilitating their direct use in biomass hydrolysis with higher protein loadings.

Expression of cell wall hydrolyzing enzymes in plant cells could confer useful agronomic traits, especially enhanced defence against plant pathogens. For example, the pectate lyase transplastomic plants showed enhanced resistance to bacterial pathogen *E. carotovora.* These results support the hypothesis that expression of pectate lyase induces plant defence responses. An early recognition of the pathogenic attack is important for successful plant defence. The pectate lyase released from chloroplast should have initiated early recognition through the formation of OG elicitors from cell wall pectin. It has been known that when pectate lyase enzymes were liberated from cells get in contact with pectin results in the release of OGs, which confers plant defence responses (Wegener and Olsen, 2004). OGs in turn induce expression of several genes involved in plant defence (Casasoli *et al.*, 2008; Ferrari *et al.*, 2007) and may be regarded as host associated molecular patterns involved in the innate immunity (Stern *et al.*, 2006; Taylor and Gallo, 2006).

The hydrolytic effectiveness of a multienzyme complex in the process of lignocellulose saccharification depends both on efficiency of individual enzymes and their ratio in the multienzyme cocktail. So far no model system of enzyme cocktails for the hydrolysis of lignocellulosic biomass has been developed that has the flexibility to optimize different enzyme classes. To the best of our knowledge, this is the first study using enzyme cocktails expressed in plants for hydrolysis of lignocellulosic biomass to produce fermentable sugars with greater flexibility to manipulate the cocktail depending upon the composition of the biomass. For example, in this study when we used xylanase and acetyl xylan esterase or pectate lyases for the hydrolysis of filter paper, no detectable sugar was released (data not shown). On the other hand, when xylan and acetyl xylan esterase or pectate lyases were used for pine wood and citrus peel hydrolysis, respectively, detectable amount of glucose were released as wood and citrus peel has significant amounts of xylan or pectin. Our study has developed a new platform for creation of chloroplast-derived enzyme cocktails for digestion of different kinds of lignocellulosic biomass.

Because lignocellulosic wastes are composed of a complex of multiple intertwined polymers, simultaneous presence of multiple hydrolases that can increase the access of each other will be required to get efficient release of monomers. Majority of enzymatic hydrolysis studies on natural substrates like pretreated wood, corn stover or wheat straw have used commercially available enzymes (Merino and Cherry, 2007; Rosgaard *et al.*, 2007a) or purified recombinant enzymes spiked with purified commercial enzymes (Gusakov *et al.*, 2007; Selig *et al.*, 2008). Accurate comparison of crude extract enzyme cocktails with commercial cocktails is not possible because of their unknown enzyme compositions. We used equivalent enzyme units based on CMC hydrolysis as a basis for general comparison and to approximately calibrate enzyme dose. Chloroplast-derived enzyme cocktail yielded 3625% and 261% more glucose equivalent units for filter paper and citrus peel, respectively, than Novozyme 188 cocktail with equivalent enzyme units; no glucose was released by Novozyme 188 from pine wood. Chloroplast-derived enzyme cocktail yielded 396%, 684% and 69% more

glucose equivalent units for filter paper, pine wood and citrus peel, respectively, than Celluclast 1.5L cocktail with equivalent enzyme units. A major drawback of submerged fermentation technology used in commercial cocktails is that the amount or composition of different enzymes can not be manipulated at will for hydrolysis of different biomass. According to Novozymes, a careful design of a combination of single component enzymes is necessary for rational utilization of these enzyme cocktails (Rosgaard *et al.*, 2007b).

Commercial production of enzymes in fermentation systems is limited by both higher cost and lower production capacity. Both these concerns are addressed by chloroplast-derived enzyme cocktails. According to NC State University Burley Tobacco Guide 2009, the cost of production of Burley tobacco in 2008 was $3506.35 per acre. Based on enzyme activity observed in plant crude extracts in this study, there is no need for purification. Therefore, enzymes could be produced as low as 0.003 cents for CelD, 0.007 cents for PelB and 0.005 cents for PelD per enzyme unit (as defined in the commercial source Megazyme). This is 3100-fold and 1057–1480-fold less expensive than endoglucanase and for pectate lyase B & D, respectively, when compared with current recombinant/purified commercial enzymes produced via fermentation (endoglucanase and pectate lyase from Megazyme). While this cost or yield comparison may not be the same for all chloroplast-derived enzymes, this concept provides a promising new platform for inexpensive enzyme cocktails to produce fermentable sugars from lignocellulosic biomass.

Experimental procedures

Isolation of genes and construction of plastid transformation vectors

Genomic DNA of *C. thermocellum* and *T. reesei* was obtained from ATCC and used as template for the amplification of different genes. Gene-specific primers using a forward primer containing a *Nde*I site and a reverse primer containing a *Xba*I site for cloning in the pLD vector were designed for *cel*D, *cel*O and *lip*Y genes. The mature region (without signal peptide) of cellulase genes *cel*D (X04584) and *cel*O (AJ275975) was amplified from genomic DNA of *C. thermocellum*. *Lip*Y (NC_000962) was amplified from genomic DNA of *M. tuberculosis*. Overlapping primers were designed for the amplification of various exons of *egl* (AB003694), *swo*I (AJ245918), *axe*1 (Z69256), *xyn*2 (X69574) and *bgl*1 (U09580) from genomic DNA of *T. reesei*. Full length cDNA of these genes was amplified from different exons by a PCR-based method (An *et al.*, 2007) using the forward of first exon and reverse of last exon containing a *Nde*I site and *Xba*I site, respectively. Pectate lyase genes *pel*A, *pel*B & *pel*D from *F. solani* with similar restriction sites including sequence for the His Tag were amplified using gene-specific primers from pHILD2A, pHILD2B (Guo *et al.*, 1995) and pHILD2D (Guo *et al.*, 1996), respectively. A similar strategy was used to amplify cutinase gene (Soliday *et al.*, 1984) from recombinant clone of *F. solani*. All the full length amplified products were ligated to pCR Blunt II Topo vector (Invitrogen) and were subjected to DNA sequencing (Genewiz). Each gene cloned in Topo vector was digested with *Nde*I/*Xba*I and inserted into the pLD vector (Daniell *et al.*, 1998, 2001) to make the tobacco chloroplast expression vector.

Regeneration of transplastomic plants and evaluation of transgene integration by PCR and Southern blot

Nicotiana tabacum var. Petite Havana was grown aseptically on hormone-free Murashige and Skoog (MS) agar medium containing 30 g/L sucrose. Sterile young leaves from plants at 4–6 leaf stages were bombarded using gold particles coated with vectors pLD-PelB, pLD-PelD and pLD-CelD and transplastomic plants were regenerated as described previously (Daniell *et al.*, 2005; Verma *et al.*, 2008). Plant genomic DNA was isolated using Qiagen DNeasy plant mini kit (Valencia, CA, USA) from leaves. PCR analysis was performed to confirm transgene integration into the inverted repeat regions of the chloroplast genome using two sets of primers 3P/3M and 5P/2M, respectively (Daniell *et al.*, 2001). The PCR was performed as described previously (Daniell *et al.*, 2001; Verma *et al.*, 2008). Leaves from the PCR positive shoots were again cut into small pieces and transferred on RMOP (regeneration medium of plants) medium containing spectinomycin for another round of selection and subsequently moved to MSO (MS salts without vitamins and growth hormones) medium containing spectinomycin for another round of selection to generate homoplasmic lines. Southern blot analysis was performed to confirm homoplasmy according to lab protocol (Kumar and Daniell, 2004). In brief, total plant genomic DNA (1–2 μg) isolated from leaves was digested with *Sma*I and hybridized with ^{32}P α[dCTP] labelled chloroplast flanking sequence probe (0.81 kb) containing the *trn*I-*trn*A genes. Hybridization was performed by using Stratagene QUICK-HYB hybridization solution and protocol.

Immunoblot analysis

Approximately 100 mg of leaf was ground in liquid nitrogen and used for immunoblot analysis as described previously (Kumar and Daniell, 2004). Protein concentration was determined by Bradford protein assay reagent kit (Bio-Rad, Hercules, CA, USA). Equal amounts of TSP were separated by SDS-PAGE and transferred to nitrocellulose membrane. The transgenic protein expression was detected using polyclonal serum raised against PelA in rabbit.

Escherichia coli enzyme (crude) preparation

Escherichia coli strain (XL-10 gold) harbouring chloroplast expression vectors expressing rCelD, rEg1 (EC 3.2.1.4), rCelO (EC 3.2.1.91), rXyn2 (EC 3.2.1.8), rAxe1 (EC 3.1.1.72), rBgl1 (EC

3.2.1.21), rCutinase (EC 3.1.1.74), rLipY (lipase, EC 3.1.1.3), rPelA, rPelB, rPelD (EC 4.2.2.2) or rSwo1 was grown overnight at 37 °C. Cells were harvested at 4 °C and sonicated four times with 30 s pulse in appropriate buffer (50 mM sodium acetate buffer with pH 5.5 for CelD, Eg1, CelO, Swo1, Xyn2, Axe1, Bgl1, 100 mM Tris–Cl with pH 7.0 for cutinase, lipase, PelA, PelB and PelD) containing protease inhibitor cocktail (Roche, Indianapolis, IN, USA) and sodium azide (0.02%). Supernatant was collected after centrifugation at 16 000 ***g*** for 10 min and protein concentration was determined.

Enzyme preparation from tobacco transplastomic leaf material

Fresh green leaves were collected and ground in liquid nitrogen. TSP was extracted in 50 mM sodium acetate buffer, pH 5.5 for cpCelD, cpXyn2 or 100 mM Tris–Cl buffer, pH 7.0 for PelD and PelB. Each enzyme used in this study (from *E. coli* and transplastomic plants) were tested for activity using suitable substrate(s). Different parameters and substrates used in these assays are given in Table 2. Protein extraction from untransformed plants was also performed under similar conditions. All buffers contained protease inhibitor cocktail (Roche) and sodium azide (0.02%). TSP was filtered using 0.22 μm syringe filter. Protein concentration (mg/mL) in TSP was spectrophotometrically determined using Bradford method at 595 nm absorption, after subtracting the turbidity of extracts at 700 nm.

Enzyme assays for pectate lyase B and pectate lyase D

Pectate lyases B and D were assayed spectrophotometrically by measuring the increase in A_{235} (Crawford and Kolattukudy, 1987; Gonzalez-Candelas and Kolattukudy, 1992; Guo *et al.*, 1995). Kinetics of the pectate lyase B and D were studied in a reaction mixture containing 1 mL of 50 mM Tris–HCl buffer (pH 8.0) with 1 mM $CaCl_2$ (freshly prepared), 1 mL of 0.0–2.5 mg/mL sodium polygalacturonate (Sigma, St Louis, MO, USA) and 0.5 mL of suitably diluted enzyme solution. Measurements were carried out at 40 °C. One unit of enzyme was defined as the amount of enzyme which forms 1 μmol of product per min with a molar extinction coefficient of 4600 per μmol/cm. Kinetic parameters (K_m and V_{max}) were calculated using nonlinear regression using Graphpad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). The initial slope of each substrate concentration was calculated, whereas the velocity (units/mg/min) was defined through the release of unsaturated galacturonic acid. The temperature optimization for pectate lyase B and D activity was carried out in 50 mM Tris–HCl buffer, pH 8.0 with or without 1 mM $CaCl_2$ at different temperatures ranging from 30 to 70 °C. In each case, the substrate was pre-incubated at the desired temperature for 5 min. To study the thermal stability of the enzyme, buffered enzyme samples were incubated for fixed time period at different temperatures. The pH optimum of the pectate lyase B and D was measured at 40 °C using different buffers like 50 mM phosphate buffer (pH 6–7), 50 mM Tris–HCl buffer (pH 8), 50 mM glycine/NaOH buffer (pH 9) or 50 mM 3-(Cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer (pH 10.0) with 2.5 mg substrate and 4 μg of TSP of PelB and PelD from both plant and *E. coli.*

Enzyme assay for CelD and commercial cocktail (Celluclast 1.5L and Novozyme 188)

Cellulase enzyme activity of cpCelD was determined by incubating crude extract in 2% carboxymethylcellulose, 5% Avicel and 5% Sigmacell (Sigma) as substrate according to IUPAC recommendations (Ghose, 1987) in 50 mM sodium acetate buffer pH 6.0, 10 mM $CaCl_2$ and incubated at 60 °C for 30 min for CMC and 2 h for Avicel and Sigmacell. Similar conditions were used in determining the pH and temperature activity profile of cpCelD using 2% CMC. Relative activity (%) was measured with reference to maximum activity obtained with 25 μg/mL for cpCelD and 10 μg/mL for rCelD. Enzyme units of commercial cocktails Celluclast 1.5L and Novozyme 188 were determined using 2% CMC, under identical assay conditions. Reducing sugar amount was determined using 3,5-dinitrosalicylic acid (Miller, 1959). D-glucose and D-galacturonic acid were used as standard to measure release of glucose equivalents and unsaturated galacturonic acid molecules. One unit of enzyme was defined as the amount of enzyme that released 1 μmole glucose equivalents per minute/mL. Cellulase unit calculation for Avicel and Sigmacell was based on glucose hexokinase method according to the manufacturer's protocol (Sigma).

In planta assay for resistance to *Erwinia* soft rot

To verify the resistance of PelB and PelD, control and transplastomic leaves were inoculated with bacterial suspension culture. *Erwinia carotovora* strain was obtained from Dr Jerry Bartz's laboratory (University of Florida, Gainesville) and grown for 24 h at 25 °C in 5 mL of LB medium. Different dilutions of bacterial cells were prepared. Five- to seven-mm areas of green house grown untransformed, PelB and PelD transplastomic tobacco leaves were scraped with fine-grain sandpaper and 20 μL of 10^8, 10^6 10^4 and 10^2 of *Erwinia* cells were inoculated to each prepared area. In a parallel study, 20 μL of the same dilutions of *Erwinia* cells were injected into leaves of untransformed, PelB and PelD transplastomic tobacco using a syringe with a precision glide needle. Photos were taken 5 days after inoculation.

Enzymatic hydrolysis of filter paper, pine wood and citrus peel

Enzyme assays were carried out either with one enzyme component or as cocktail on filter paper, pine wood and orange peel, and released reducing sugar was determined using DNS method. Orange peel prepared from Valencia orange (*Citrus sinensis* cv Valencia) fruit and albedo portion was air dried overnight and ground in liquid nitrogen. Ground Valencia orange peel, albedo portion and pine wood biomass were washed several times in distilled water until no reducing sugar was detected by DNS reagent as well as by glucose hexokinase method.

For enzymatic digestion, 50–200 mg of filter paper, pine wood sample or ground orange peel was used. Enzyme catalytic function of chloroplast-expressed enzymes like Eg1, CelO, Bgl1, Swo1, Xyn2, PelA and cutinase were tested using known amount of crude extract TSP with appropriate substrates before making suitable enzyme

cocktail. Filter paper activity was determined using Whatman No. 1 filter paper strip at pH 5.5 and 50 °C. Different combinations of crude extracts contained TSP in the range of 25–200 μg/mL. rEg1 (100 μg/mL), rBgl1 (200 μg/mL), rSwo1 (120 μg/mL), rCelO (100 μg/mL), cpCelD (100 μg/mL), cpEg1 (100 μg/mL), cpBgl1 (200 μg/mL), cpSwo1 (25 μg/mL) and cpCelO (100 μg/mL) were used. Untransformed leaf and *E. coli* crude extracts contained 485 μg TSP. The samples were incubated in 50 mM sodium acetate buffer plus 10 mM $CaCl_2$, 20 μg BSA for 36 h. Hydrolysis of pine wood sample was carried out (pH 5.5–8.0, 40–50 °C, 36 h incubation) using a cocktail of crude extracts containing TSP in the range of 50–250 μg/5 mL. cpPelB (250 μg), cpPelD (250 μg) (at pH 8.0,), cpCelD (200 μg), cpXyn2 (200 μg), rEg1 (100 μg), rBgl1(200 μg), rSwo1 (120 μg), rCelO (100 μg), rAxe1 (100 μg), rPelA (200 μg), rCutinase (50 μg) and rLipY (100 μg) were used in the cocktail. Untransformed leaf and *E. coli* crude extracts contained 1550 μg TSP/5 mL. Hydrolysis of Valencia orange peel (200 mg/5 mL reaction, pH5.5–8.0, 40–50 °C, 36 h incubation) was carried out using a cocktail of crude extracts containing TSP in the range of 50–250 μg/5 mL. cpPelB (250 μg), cpPelD (250 μg), cpCelD (100 μg) and cpXyn2 (100 μg), rEg1 (100 μg), rBgl1(200 μg), rSwo1(120 μg), rCelO (100 μg) and cpCelD (100 μg), rAxe2 (100 μg), rCutinase (50 μg), rLipY (100 μg), rPelA (200 μg) were used in the cocktail. Untransformed leaf and *E. coli* crude extracts contained 1670 μg TSP/5 mL. All experiments were carried out under specified conditions in a rotary shaker at 150 r.p.m. Crude extracts containing enzymes from *E. coli* and plants were used in the cocktail for hydrolysis. End product reducing sugar was determined using DNS reagent (Miller, 1959) with D-glucose and D-galacturonic acid as standard. Ampicillin and kanamycin 100 μg/mL was added to prevent any microbial growth during the long durations of enzyme hydrolysis. Commercial enzyme cocktails Celluclast 1.5L and Novozyme 188 were tested for hydrolysis of citrus peel and pine wood in the same assay conditions used for enzyme cocktails from crude extracts. Enzyme units of Celluclast 1.5L and Novozyme 188 used for hydrolysis assays were equivalent to cpCelD enzyme units (based on CMC hydrolysis) present in cocktails of crude extracts. In all experiments, control assays contained substrate without enzyme or enzyme without substrate. All experiments and assays were carried out in triplicate.

Acknowledgement

The investigations reported in this article were supported in part by grants from USDA 3611-21000-021-02S , USDA CSREES 2009-39200-19972 and NIH R01 GM 63879 to Henry Daniell. Authors are thankful to Dr Velpula for optimization of pectate lyase assays, Dr Jerry Bartz (University of Florida, Gainesville) for providing *E. carotovora* culture and Dave Litzen (KL Energy Corporation) for providing pine wood samples.

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